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A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases

(DNA repair/phosphodiesterase/*Saccharomyces cerevisiae*/camptothecin)

SHU-WEI YANG, ALEX B. BURGIN, JR., BETH N. HUIZENGA, CAROL A. ROBERTSON, KEVIN C. YAO, AND HOWARD A. NASH

Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD 20892-4034

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ABSTRACT **The covalent joining of topoisomerases to DNA is normally a transient step in the reaction cycle of these important enzymes. However, under a variety of circumstances, the covalent complex is converted to a long-lived or dead-end product that can result in chromosome breakage and cell death. We have discovered and partially purified an enzyme that specifically cleaves the chemical bond that joins the active site tyrosine of topoisomerases to the 3′ end of DNA. The reaction products made by the purified enzyme on a variety of model substrates indicate that the enzyme cleanly hydrolyzes the tyrosine–DNA phosphodiester linkage, thereby liberating a DNA terminated with a 3′ phosphate. The wide distribution of this phosphodiesterase in eukaryotes and its specificity for tyrosine linked to the 3′ end but not the 5′ end of DNA suggest that it plays a role in the repair of DNA trapped in complexes involving eukaryotic topoisomerase I.**

Topoisomerases are ubiquitous enzymes that play important roles in virtually all cells. The defining activity of these enzymes is their capacity to break DNA and, after an interval in which topological changes may occur, to reseal the break without the intervention of a high energy cofactor. Reversible breakage and reunion by topoisomerases is achieved through an intermediate in which a specific tyrosine residue of the enzyme is covalently joined to the DNA at the site of the break. The tyrosine–phosphodiester linkage is formed with one of two distinct polarities. Some enzymes join to the 5′ end of the break, liberating a fragment terminated by a 3′ hydroxyl; representatives include bacterial topoisomerase I as well as DNA gyrase and its relatives, the topoisomerase II family. Other topoisomerase enzymes join to the 3′ end of the break, liberating a fragment with a free 5′ hydroxyl; representatives include the eukaryotic type I topoisomerases and the Int family of site-specific recombinases (for reviews, see refs. 1 and 2).

The covalent intermediate between a topoisomerase and DNA is normally quite transient, i.e., strand rejoining is fast relative to strand cleavage. However, when a topoisomerase acts on DNA that contains imperfections (3, 4) or when specific inhibitors are present (5, 6), the rejoining step is slowed or blocked. This shifts the covalent complex from a transient intermediate toward a dead-end product; the associated long-lived break in the DNA backbone can have dire consequences for chromosome stability and cell survival (5, 6). In this work, we describe an activity that hydrolyzes the covalent bond that joins topoisomerases to the 3′ end of DNA. The specificity, robustness, and widespread distribution of this enzyme suggest that it could be an important element in the repair of DNA that has become stuck in covalent linkage with eukaryotic type I topoisomerases.

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MATERIALS AND METHODS

DNA Substrates. Conventional oligonucleotides were constructed using an Applied Biosystems model 391 DNA synthesizer (Perkin–Elmer) or were purchased from Midland Certified Reagent (Midland, TX). An oligonucleotide comprising the sequence of the top strand of the *att*B of *Escherichia coli* from positions −20 to −3 (7) was constructed, as described (8), by conventional nucleotide synthesis on a resin derivatized with a protected tyrosine moiety. The 3′ terminus of the resulting 18 mer, oHN279Y, consists of a phosphate in ester linkage with a tyrosine moiety (Fig. 1*C*). Because release of the oligonucleotide from the resin involves treatment with ammonium hydroxide, it is likely that the tyrosine carboxyl is converted to a carboxylamide (9), but for simplicity, we refer to the synthesis product as a phosphotyrosine oligonucleotide. An oligonucleotide with the identical sequence but containing a phosphotyrosine at the 5′ end was constructed on the same tyrosine resin using nucleotide synthons (Glen Research, Sterling, VA) that permit synthesis in the 5′-to-3′ direction.

All oligonucleotides were purified after removal (where relevant) of the 5′ trityl group either by reversed-phase HPLC followed by lyophilization, essentially as described (10), or by electrophoresis with a urea/16% polyacrylamide gel followed by electroelution. The 3′ and 5′ ends of oligonucleotide substrates were labeled with, respectively, [α-^{32}P]ddATP (Amersham) plus terminal deoxynucleotide transferase (Pharmacia) and [γ-^{32}P]ATP (DuPont/NEN) plus either T4 polynucleotide kinase (New England Biolabs) or a mutant version of it (Boehringer Mannheim) that has greatly reduced 3′ phosphatase activity. Unincorporated label was removed by passage through a Bio-Spin P6 column.

Duplex substrates were prepared by mixing oligonucleotides in 50 mM NaCl and 1 mM sodium EDTA, heating to 80° and cooling slowly to room temperature. Duplexes involving the 3′ tyrosine oligonucleotide (oHN279Y) employed oligonucleotide oHN274, comprising the bottom strand of *att*B from positions −19 to +22 and either oHN282, comprising the top strand of *att*B from positions +1 to +21 or oHN289, comprising the top strand of *att*B from positions −2 to +21. A duplex with a single strand "flap" was made by annealing oHN274, oHN289, and oHN290, a 28-mer in which 10 nucleotides of irrelevant sequence (5′GCATGCACGT) follows the sequence of the *att*B 18-mer.

Covalent complexes between Int protein and DNA were made essentially according to the method of Nunes-Düby *et al.* (11). A suicide substrate with a nick 3 nucleotides adjacent to a site of Int cleavage was assembled by annealing oHN274, oHN282, and oHN281, the latter comprising the top strand of *att*B from positions −20 to 0. This substrate was included at a concentration of 120 nM in a reaction mixture (50 μl) containing 50 mM Tris·HCl (pH 8.0), 80 mM KCl, 5% glycerol, 2 mM EDTA, 1 mM mercaptoethanol, and ≈10 μM purified Int protein. The mixture was incubated at 25°C for 30–120 min and, unless otherwise specified, subsequently heated to 65°C

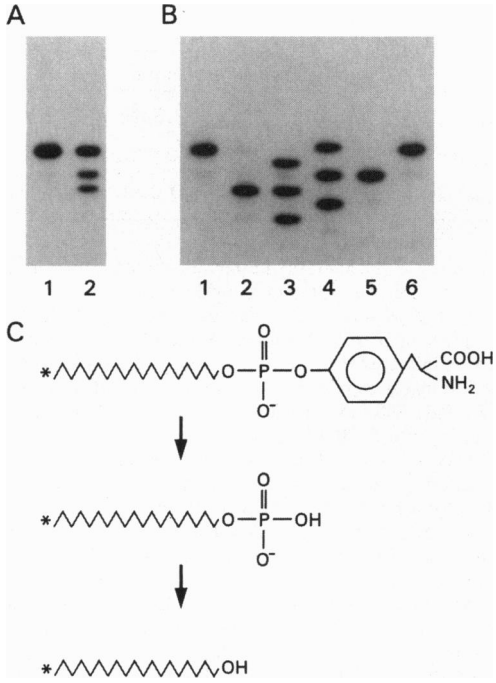

FIG. 1. Enzymatic processing of an oligonucleotide containing a tyrosine–phosphodiester. (*A*) An 18 mer, oHN279Y, radiolabeled at its 5′ end, and terminated at its 3′ end with phosphotyrosine, was incubated at 30°C for 20 min with either extraction buffer (lane 1) or a cellular extract of *S. cerevisiae* (lane 2) and analyzed by urea/PAGE. Preparation and labeling of the substrate as well as reaction conditions are given in *Materials and Methods*. (*B*) The radiolabeled substrate was incubated with 5 μl of either enzyme dilution buffer (lanes 1 and 6) or a 5,000-fold dilution of a partially purified enzyme preparation (lanes 2 and 5). After 20 min at 30°C, the reactions of lanes 5 and 6 were brought to 10 mM with $MgCl_2$ and supplemented with 15 units of T4 polynucleotide kinase; incubation was continued at 37°C for 20 min before urea/PAGE. Lanes 3 and 4 contain, respectively, 3′-phosphorylated and nonphosphorylated oligonucleotide markers: equimolar mixtures of 17, 18, and 19 mers, each radiolabeled at its 5′ end. (*C*) Schematic of the proposed enzymatic transformation. The jagged line represents the 18-mer oligonucleotide with an asterisk at its left to indicate the radiolabeled 5′ end. At the right of the jagged line, the chemical structure of the 3′ end of the oligonucleotide is given.

for 10 min. Covalent complexes between DNA and eukaryotic topoisomerase I were made using a suicide half-site duplex containing a preferred cleavage site for this enzyme (12). This was made by annealing oligonucleotides oHN205 and oHN206 (ref. 8); incubation with purified calf thymus topoisomerase I (a gift from L. Liu, Robert Wood Johnson Medical School, Piscataway, NJ) was essentially as described (8).

Chromogenic phosphodiesterase substrates *p*-nitrophenyl phosphate and *p*-nitrophenyl thymidine 5′ phosphate were purchased from Sigma. A sample of *p*-nitrophenyl thymidine 3′ phosphate was synthesized by S. Beaucage (Center for Biologies and Evaluation, Bethesda), essentially as described (13).

Preparation of Phosphodiesterase. *Saccharomyces cerevisiae* strain JN284 was grown at 30°C in YPDA medium (14). Small-scale extracts (≈0.9 ml; ≈1.0 mg/ml of protein) were made from 12.5 ml of cells that had been grown to an OD_{600} of ≈1, washed once with water, resuspended in 330 μl of disruption buffer [20 mM Tris·HCl (pH 8.0) containing 30 mM ammonium sulfate, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5% glycerol, and two cocktails (15) of protease inhibitors], disrupted by 5 cycles of shaking with glass beads and centrifuged for 30 min at 4°C. Large-scale extracts were prepared with the cells from a 100-liter culture (prepared by Y. Shiloach, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda) that had been grown to an OD_{600} of ≈9. These were resuspended in 3.8 liters of ice cold buffer H [50 mM Tris·HCl (pH 8.0) containing 0.2 M NaCl, 10 mM mercaptoethanol, 3 mM EDTA, 10% glycerol, and 2 mM phenylmethylsulfonyl fluoride], lysed by three passages at 9000 psi through a French press, and centrifuged in a Sorvall GS-3 rotor at 8200 rpm for 10 min. To the supernatant (≈4.0 liters; ≈14 mg/ml of protein) was added 0.05 vol of a 10% solution of polyethyleneimine (BDH; adjusted to pH 7.9 with HCl). After stirring and centrifugation the supernatant was precipitated with ammonium sulfate (45–80% saturation) and dialyzed against buffer C [50 mM Tris·HCl (pH 8.0) containing 10% glycerol, 0.1 M NaCl, 5 mM mercaptoethanol, 1 μg/ml phenylmethylsulfonyl fluoride, and 2 mM EDTA] to yield a preparation (2.2 liters) containing ≈38 g of protein.

The entire large-scale extract was subjected to chromatography with several commercially available separation media; in each case, column fractions were assayed for release of tyrosine from radiolabeled oligonucleotide oHN279Y as described below. Several of these trials failed to substantially improve enzymatic purity, in some cases simply because the columns were overloaded with protein. Thus, a definitive purification of the activity remains to be established. Nevertheless, substantial purification was achieved with S-Sepharose (Pharmacia; activity eluted at ≈0.25 M NaCl), double-stranded DNA cellulose (Sigma; activity eluted at ≈0.18 M NaCl), and monoS (Pharmacia; activity eluted at ≈0.35 M NaCl). We estimate that, taken together, the purification steps resulted in at least a 5000-fold increase in specific activity. The final material was dialyzed against buffer S [50 mM Tris·HCl (pH 8.0) containing 0.2 M NaCl, 2 mM EDTA, 4 mM mercaptoethanol, and 40% glycerol]. This preparation (≈4.5 ml; ≈2.2 mg/ml protein) shows a complex pattern of bands upon SDS/PAGE; it was stored at −20°C and, unless otherwise noted, was used for all the experiments in this work.

Enzyme Assays. The standard phosphodiesterase assay was performed by assembling a premixture (6 μl) that contained 100 mM Tris·HCl (pH 8.0), 160 mM KCl, 4 mM sodium EDTA, 2 mM DTT, 10% glycerol, 80 μg/ml BSA, and 0.04 pmol of radiolabeled substrate. To this was added either 6 μl of crude extract in disruption buffer (see above) or 4 μl of water plus 2 μl of partially purified enzyme in enzyme dilution buffer [50 mM Tris·HCl (pH 8.0) containing 100 mM NaCl, 5 mM sodium EDTA, 10% glycerol, 5 mM DTT, and 500 μg/ml BSA]. Unless otherwise indicated, reactions were incubated at 30°C for 20 min and analyzed by electrophoresis on a 12% sequencing gel. In some cases, the mobility of substrate and products was compared with that of a set of marker oligonucleotides, terminated either with 3′ hydroxyl or 3′ phosphate moieties, whose sequence comprised the top strand of *att*B from −20 to either −4, −3, or −2 (Midland Certified Reagent).

For reactions with a covalent complex, the tyrosine-oligonucleotide substrate was replaced in the standard assay with 3.5–5.0 μl of the suicide substrate reaction mixture described above. After incubation with phosphodiesterase at 30°C for various times, reactions were either analyzed as above or on an 8% polyacrylamide gel containing 4 M urea and 0.1% SDS.

For reactions with a nitrophenol compound, the standard premixture was scaled up 8.33-fold, omitting glycerol and the tyrosine-oligonucleotide. Chromogenic substrate was then added to a concentration of 5.55 mM; to this mixture (90 μl) was added 10 μl of undiluted enzyme. After 16 hr at 30°C, 0.9 ml of 0.1 M NaOH was added and absorbance was read at 400 nm against a control in which enzyme was replaced with buffer S.

RESULTS AND DISCUSSION

Characterization of Enzyme Activity with an Oligonucleotide-Tyrosine Substrate. Recently, Sadowski and colleagues

(16) introduced a novel substrate for use in the study of site-specific recombination. This substrate consists of a DNA fragment that is derivatized to contain a tyrosine residue in ester linkage with the phosphate at the 3′ end of the DNA chain. The tyrosine–phosphodiester thus recapitulates the chemistry found at the covalent joint between DNA and either Int-family recombinases or eukaryotic type I topoisomerases. We modified the original method for preparing these substrates and used the resulting material to study the details of Int-promoted reactions of DNA (8). During the course of our work, we noted that the substrate was modified in unexpected ways during routine treatment with commercial sources of terminal deoxynucleotidyl transferase. However, a sample of highly purified terminal transferase (a generous gift from M. S. Coleman, University of North Carolina, Chapel Hill) lacked the modification activity, suggesting the existence of a separate enzyme. To study the modification activity, we focused on one version of the novel substrate (Fig. 1); incubation of it with crude extracts from the budding yeast *S. cerevisiae* resulted in production of two discrete species of slightly higher mobility. In addition to yeast, crude extracts (described in ref. 17) from a variety of eukaryotic sources (*Drosophila melanogaster* embryos, a mouse/rat neuroblastoma-glioma hybrid cell line, and a human mammary tumor cell line) generated one or both novel species upon incubation with this substrate (data not shown). In contrast, little or no activity could be detected in extracts of *E. coli*.

To characterize the enzymatic activity further we subjected the crude yeast extract to column chromatography. Because preliminary data (not shown) indicated that it was the simplest derivative of the substrate, we focused on fractions that produced the faster-moving species. The result was a partially purified enzyme preparation that, as shown in Fig. 1*B*, can achieve nearly quantitative conversion of the tyrosine-oligonucleotide substrate to a unique product. Comparison with a ladder of suitably prepared standards shows that this species has the mobility expected from the hydrolytic loss of the terminal tyrosine. This view is supported by the effect (Fig. 1*B*) of an enzyme known to remove phosphate groups from the 3′ end of DNA, the kinase/phosphatase encoded by bacteriophage T4 (18). In contrast, little or no conversion was seen following treatment with a mutant form of this enzyme that is specifically deficient in phosphatase activity (data not shown). It thus appears that, of the two principal products formed from the model substrate by crude extracts (Fig. 1*A*), the one with faster mobility results from the action of the enzyme we have purified while that with the slower mobility represents subsequent action by a phosphatase in the crude extract. The kinetics of appearance of these two products supports this assertion (data not shown). We preliminarily describe the partially purified enzymatic activity as a tyrosine–DNA phosphodiesterase (Fig. 1*C*). To our knowledge, this is the first description of such an enzyme.

Although not characterized in detail, the partially purified enzyme is active under a wide variety of conditions, e.g., at pH values ranging from 5.0–9.5 and at temperatures from 25–45°C. In addition, there appears to be no cofactor requirement: reactions are carried out in a simple buffer and no stimulation of activity is observed when reactions are supplemented with 10 mM Mg, Ca, Co, Cd, or Zn. The dependence of enzyme activity on substrate concentration fits well to a rectangular hyperbola, yielding a K_m value (Fig. 2) in the nM range. Activity of the partially purified enzyme is destroyed by brief heating at temperatures above 65°C and by treatment with proteinase K but not by treatment with RNase A. All of the above results suggest that enzymatic activity is contained in a relatively uncomplicated protein. This inference is supported by gel exclusion chromatography of the partially purified material; enzymatic activity elutes with an apparent molecular mass of ≈55 kDa (Fig. 3).

	Single-strand	Gapped	Nicked
K_m	8.8nM	2.3nM	2.7nM
k_{cat} (rel)	≡1.0	0.16	0.04
k_{cat}/K_m	0.113	0.069	0.014

FIG. 2. Kinetic parameters of enzyme activity. Substrates with the identical 18-mer tyrosine-oligonucleotide were prepared as described in *Materials and Methods* either as the single-stranded form, as a gapped duplex, or a nicked duplex. A radiolabeled version of each substrate was mixed with a suitable amount of unlabeled material to yield reaction mixtures with final substrate concentrations of 1.2, 3.3, 11.7, 28.3, and 70.0 nM. Following incubation with the partially purified enzyme for 5–20 min and analysis by urea/PAGE, the initial rate of conversion of the radiolabeled substrate to the 3′-phosphorylated product was determined with a PhosphorImager (Molecular Dynamics). The data were fit into the classic Michaelis–Menten equation to yield estimates of K_m and V_{max}; for each substrate, the standard error for these two estimates was <30% and <5% of the computed value, respectively. The value for V_{max} was converted to a specific activity by dividing by the concentration of enzyme protein used in the experiment. For each substrate this value was expressed relative to that obtained for the single-strand substrate (0.029 nmol/min/μg), yielding a relative value for k_{cat}. The data shown are for a single experiment; for each substrate, an independent experiment performed at enzyme concentrations that differed by at least 3-fold from those in the experiment in this figure gave very similar values.

Enzyme Activity on Duplex Substrates. Type I topoisomerases typically act on duplex DNA (1, 2). To fashion model substrates that more closely resemble natural topoisomerase intermediates, we annealed the tyrosine-containing oligonucleotide described above to a set of appropriate strands to make two types of duplex substrate. These are distinguished by what lies to the 3′ side of the nucleotide derivatized by tyrosine: a 3-nt gap in the first substrate and a nick in the second. These alternatives model two different scenarios for trapping of a

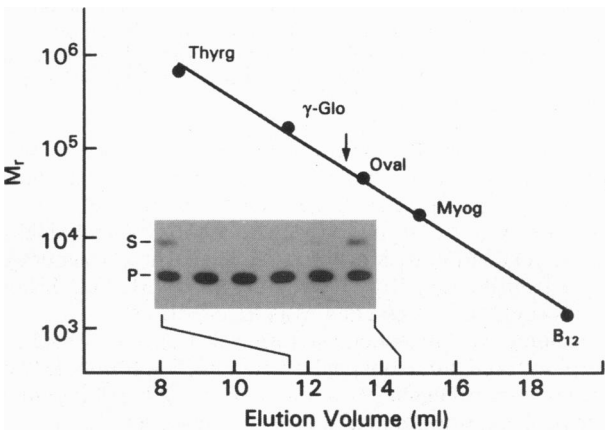

FIG. 3. Size fractionation of enzymatic activity. A sample (100 μl) of partially purified enzyme was loaded onto a Superose 12 (Pharmacia) column (1 cm × 30 cm) that had been equilibrated with 50 mM Tris·HCl (pH 8.0) containing 10% glycerol, 0.5 M NaCl, 5 mM mercaptoethanol, 2 mM EDTA, and 1 μg/ml phenylmethylsulfonyl fluoride. The column was eluted with the same buffer; 0.5 ml fractions were collected and assayed as in Fig. 1. Activity elutes as a single peak, with slight tailing toward larger elution volumes. The inset shows the conversion of radiolabeled tyrosine-oligonucleotide (S) to product (P) by aliquots of column fractions collected between 11.5 and 14.5 ml of elution. The graph compares the position of peak activity (arrowhead) relative to that of marker proteins (bovine thyroglobulin, bovine gamma globulin, chicken ovalbumin, horse myoglobin, and vitamin B12) that were chromatographed on the same column.

covalent topoisomerase complex (5, 11). As shown in Fig. 2, these substrates also have low K_m values, although their k_{cat} values are somewhat reduced. We do not know if this reduction reflects the rate of the chemical conversion of substrate to product or some other step in the reaction cycle, such as the release of product from the enzyme surface. In any case, the data of Fig. 2 show that gapped and nicked duplexes are indeed substrates for the enzyme; the relative values of k_{cat}/K_m indicate that, if presented at equal concentrations in a competition experiment, tyrosine would be removed from these substrates 58% and 11% as efficiently, respectively, as from a single-stranded substrate.

The partially purified phosphodiesterase cannot only separate a tyrosine from its covalent attachment to a duplex, it can unlink an entire topoisomerase that is joined to a duplex through an enzymic tyrosine residue. Such substrates are conveniently prepared by a "suicide" strategy. A topoisomerase is incubated with a duplex that contains a strand interruption a few nucleotides adjacent to the expected point of enzyme attack (11, 19). The resulting covalent complex is long-lived, presumably because the 5′ hydroxyl residue that would normally be used for religation has been lost from the duplex as a very short oligonucleotide, leaving a short gap adjacent to the point of covalent attachment. Fig. 4*A* shows that such complexes made using radiolabeled duplex and Int protein have a characteristic mobility in SDS/PAGE. Incubation with the partially purified phosphodiesterase leads to loss of this species (Fig. 4*A*), indicating that the enzyme has removed Int from the complex. Indeed, analysis of similar samples by urea/PAGE shows that loss of a species that is trapped at the top of the gel is correlated with the appearance of a fragment that terminates precisely at the expected point of Int attack (Fig. 4*B*, lanes 1 and 2). Moreover, this species

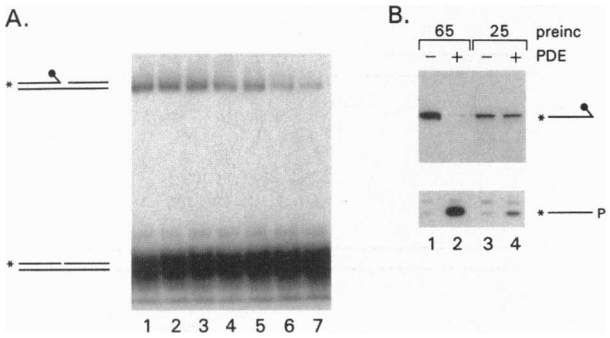

FIG. 4. Enzymatic release of Int topoisomerase from a covalent complex. (*A*) A duplex "suicide substrate" made by annealing a radiolabeled 21 mer with a set of complementary strands was incubated with purified Int protein for 2 hr as described in *Materials and Methods*, heated to 65° for 10 min and then incubated at 30° with a 30-fold dilution of the partially purified enzyme. The samples of lanes 1–7 were withdrawn after 15 sec, 30 sec, 1 min, 2 min, 4 min, 8 min, and 16 min, respectively; they were quenched and electrophoresed through an 8% polyacrylamide/4 M urea/0.1% SDS gel, as described in *Materials and Methods*. Cartoons of the suicide substrate and the covalent complex with Int are presented at the left of lane 1; these are shown as duplexes although we do not know the extent to which they are denatured during electrophoresis. (*B*) After incubation of a suicide substrate and Int for 90 min at 25°C, aliquots were incubated an additional 10 min at either 65°C (lanes 1 and 2) or 25°C (lanes 3 and 4) prior to incubation with either enzyme dilution buffer (lanes 1 and 3) or a 50-fold dilution of the partially purified enzyme (lanes 2 and 4). Samples were analyzed by urea/PAGE as in Fig. 1. Two segments of the resulting autoradiogram are shown: one is from the top of the gel showing material trapped in the wells and the other is from near the bottom of the gel. As indicated by the cartoons on the right, these contain an 18-mer oligonucleotide covalently attached to the entire Int protein and an 18-mer oligonucleotide terminated with a 3′ phosphate, respectively.

appears to end with a 3′ phosphate; treatment with T4 polynucleotide kinase/phosphatase both reduces the mobility of this species by the expected amount and permits it to serve, after annealing to a suitable complementary strand, as a primer for DNA synthesis (data not shown).

Reaction mixtures with Int complexes typically require higher concentrations of the partially purified enzyme than does the standard assay. Rather than indicating that covalent complexes are poor substrates, this probably reflects significant inhibition of the phosphodiesterase by the excess Int protein and/or suicide duplex that accompany the covalent complex. When the partially purified phosphodiesterase is incubated with a roughly equimolar mixture of the Int covalent complex prepared as in Fig. 4 and the gapped duplex substrate described in Fig. 2, tyrosine is removed from the gapped substrate at a rate only two to three fold faster than Int is removed from the covalent complex (data not shown). Although not studied in as much detail, covalent complexes between calf thymus topoisomerase I (a generous gift from L. Liu) and a preferred DNA suicide target site (12) are also readily unlinked by the partially purified phosphodiesterase.

Interestingly, the Int covalent complex is a much less effective substrate if, prior to incubation with the phosphodiesterase, it has not been heated to 65°C (Fig. 4*B*, lanes 3 and 4). Under these conditions, less DNA is present as a covalent complex (Fig. 4*B*, lanes 1 and 3) so a precise comparison of substrate reactivity is difficult. However, when higher amounts of Int are used to overcome this limitation, there is still a striking difference between the activity of the phosphodiesterase on covalent complexes that have and have not been heated (data not shown). In contrast, little or no effect of prior heating is observed with tyrosine-oligonucleotide substrate (data not shown). Similar studies have not yet been carried out with eukaryotic topoisomerase I.

Specificity of Enzyme Action. The fact that the partially purified enzyme yields a single product with a unique mobility (Fig. 1*B*) indicates that the enzyme has a much higher specificity for the tyrosine–phosphodiester than for the other phosphodiester bonds in the oligonucleotide. This specificity must be quite strong because the same unique band is seen when the enzyme concentration is raised by 100 fold over that used in Fig. 1*B* (data not shown). In addition, no novel products are observed when high concentrations of the partially purified enzyme are incubated with unmodified oligonucleotides, i.e., those that end with a 3′ hydroxyl or 3′ phosphate rather than a 3′ tyrosine. The failure to modify oligonucleotides that end in 3′ phosphate or to remove the 5′ radiolabel further indicates that the purified enzyme has neither 5′ nor 3′ phosphatase activity.

We have attempted to target the enzyme to an internal phosphodiester bond of DNA by creating a branch point between duplex and single-stranded DNA. Several enzymes thought to be involved in DNA repair, such as the RAD1-RAD10 nuclease, have relative specificity for such "flap" structures (20). The substrate, made to have a noncomplementary single-stranded branch in the middle of the duplex, was unaffected by a large excess of the partially purified enzyme, both in the presence and absence of magnesium (data not shown). This suggests that it is the chemistry of the linkage rather than a structural feature that is recognized. It would be of interest to examine enzyme activity on other DNA–amino acid linkages, such as serine–DNA or threonine–DNA phosphodiesters.

Like eukaryotic topoisomerase I, members of the topoisomerase II family also form a tyrosine–DNA phosphodiester (1, 2). However, in this case the covalent joint is to the 5′ end of DNA rather than the 3′ end. To compare enzyme activity on these two kinds of substrates, we synthesized an oligonucleotide with a sequence identical to that used in Fig. 1 but with tyrosine covalently joined to the 5′ end. However, incubation

of this oligonucleotide with the partially purified enzyme under the conditions of Fig. 1 yielded no new products (data not shown). Adding divalent metals, increasing the concentration of enzyme 100-fold or annealing the substrate to a complementary strand so as to present the tyrosine–phosphodiester as a four nucleotide 5′ extension from a duplex all failed to significantly improve the efficiency of the tyrosine removal. We conclude that the partially purified enzyme is specific for a 3′ tyrosine–phosphodiester. (A preliminary search for an activity in crude yeast extracts that hydrolyzes the tyrosine-5′ oligonucleotide has been unsuccessful.)

The specificity of the phosphodiesterase for the kind of intermediate made by type I but not type II topoisomerases was confirmed with mononucleotide substrates in which the phosphate group was esterified to nitrophenol. With these classic phosphodiesterase substrates (13, 21), detection is not of the released DNA segment but of the nitrophenol moiety, via the increase in absorbency that accompanies hydrolysis of the phosphodiester. Because of the relative insensitivity of the assay, large amounts of enzyme and long incubations were required. However, as shown in Fig. 5, thymidine 3′ nitrophenylphosphate (compound a) is an effective substrate for the partially purified enzyme but thymidine 5′ nitrophenylphosphate (compound b) is not. The absorption spectrum of the product of enzyme action on the former substrate is identical to that of authentic nitrophenol (data not shown), confirming our assumption that the action of our purified enzyme is a simple hydrolysis of the aryl–DNA phosphodiester. (Of course, in the absence of a detailed mechanism for the enzyme, we do not know if the hydrolysis is achieved in a single step or if the enzyme first cleaves the phosphodiester with an alternate nucleophile that is subsequently hydrolyzed.) Fig. 5 also shows that the partially purified enzyme is much more active as a tyrosine–DNA phosphodiesterase than as a tyrosine–phosphatase.

Selective action on thymidine 3′ nitrophenylphosphate vs. thymidine 5′ nitrophenylphosphate is a property of one well-studied enzyme, phosphodiesterase II, traditionally purified from spleen (21). However, this enzyme is thought to act by stepwise hydrolysis of mononucleotides from the 5′ end of a chain and shows no preference for an aryl–DNA phosphodiester bond over a conventional DNA phosphodiester. Indeed, commercial phosphodiesterase II readily degraded a conventional oligonucleotide that had been radiolabeled at its 3′ end. However, neither under the conditions of Fig. 1 nor under conditions described as optimal for spleen phosphodiesterase II (21) did we observe significant shortening of this substrate by the partially purified phosphodiesterase. The identity of this enzyme remains to be determined.

Implications for DNA Repair. In this work we have described the partial purification and biochemical characterization of an enzyme from yeast that can hydrolyze the covalent bond that joins a topoisomerase to the 3′ end of DNA. Of course, in the absence of *in vivo* experiments with a specific inhibitor or a mutant, we cannot be certain that separating topoisomerases from covalent complexes is an important biological function for this enzyme. For example, perhaps an aryl-phosphodiester bond is produced in a biochemical reaction not involving DNA (as in the synthesis of a novel phospholipid); the enzyme that we have described might be involved in the metabolism of this hypothetical compound. Nevertheless, for the following reasons a role for the phophodiesterase in the repair of trapped topoisomerase complexes is attractive. First, enzyme activity on a DNA–tyrosine-oligonucleotide is characterized by a half-maximal substrate concentration in the nanomolar range (Fig. 2). This indicates that the covalent complex between topoisomerases and DNA (whose essential feature is incorporated in the oligonucleotide) is readily accepted by the enzyme and thus may represent its natural substrate. Second, this proposed substrate should be generated with reasonable frequency. Eukaryotic type I topoisomerase is abundant enzyme (22) and, although the covalent complex is designed to be a transient intermediate, it is stabilized by several recurring irregularities in DNA such as mismatches, UV-induced lesions and nicks (3, 4, 11, 12, 19). Subsequent encounter with a replication fork converts the covalent complex into a double-strand break, a potentially lethal lesion (5, 6). It would therefore not be surprising to find that cells have mounted more than one kind of repair process for this kind of insult. The capacity of cells to repair double-strand breaks by homologous and illegitimate recombination is well established (23) but the enzyme described in this work offers an independent (and preventive) solution to potential topoisomerase damage. Third, the enzyme is found in organisms where the proposed substrate is most likely to occur, i.e., in eukaryotes where enzymes that

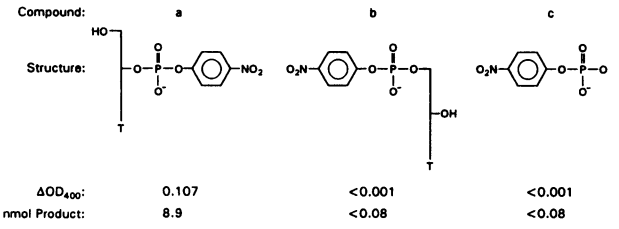

FIG. 5. Hydrolysis of p-nitrophenyl phosphodiesters. Each of the compounds diagrammed in columns a–c were incubated under identical conditions, described in *Materials and Methods*, with a high concentration of the partially purified enzyme. The change in absorbance at 400 nm (relative to identical reactions incubated with enzyme dilution buffer) is shown below the relevant diagram. The molar amount of product calculated from this value and the extinction coefficient of nitrophenol is given in the last line.

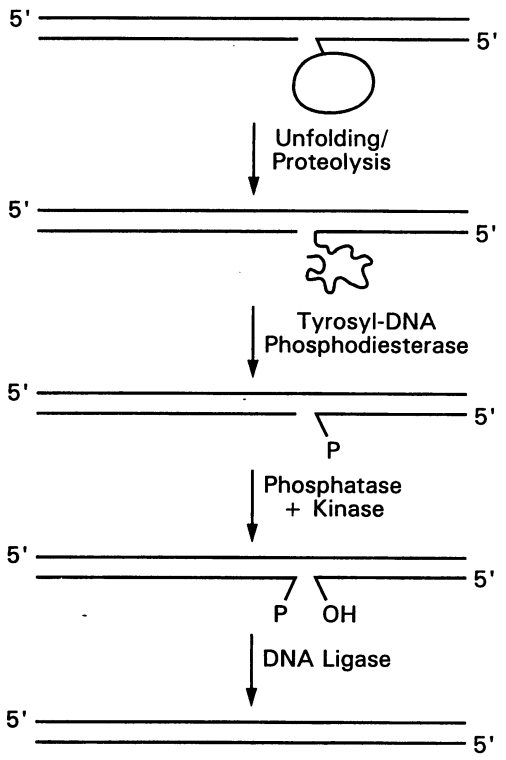

FIG. 6. A speculative pathway for repair of covalently trapped eukaryotic topoisomerase I. A duplex DNA that is interrupted by the covalent attachment by a topoisomerase to the 3′ end of one strand is shown at the top. Subsequent cartoons indicate successive steps in the conversion of the protein-linked break to a continuous duplex.

attack the 3′ end of DNA are common but not in bacteria (at least *E. coli*), where they are rare.

If covalent complexes between eukaryotic topoisomerase I and DNA are indeed important natural substrates for this enzyme, we can envision its role in a DNA repair pathway that proceeds according to the outline presented in Fig. 6. In this pathway the clean removal of protein from the DNA is the key step. Although the covalent complex can catalyze its own hydrolysis (24), the reaction is very inefficient and is only observed around pH 9. In contrast, the enzyme we have characterized is robust and active at physiological pH. Even after phosphodiesterase action, the DNA contains a nick that must be resealed so that subsequent replication does not induce a double-strand break. Prior to closure of the nick by DNA ligase, the 3′ phosphate left by the phosphodiesterase must be removed and the 5′ hydroxyl generated by the initial topoisomerase cleavage must be phosphorylated; the relevant enzymatic activities are known to be widespread in eukaryotes (ref. 25 and references therein) and there is at least one report that they copurify (26). Our data also suggest that there is an important step in the pathway prior to phosphodiesterase action. When we used Int protein as a model topoisomerase attached to the 3′ end of a DNA break, we found that it was much more active as a substrate for the phosphodiesterase after a brief heating (Fig. 4*B*). One possible explanation (suggested to us by M.-A. Bjornsti, Thomas Jefferson University, Philadelphia) is that heating serves to partially disanneal the duplex substrate, thereby presenting the scissile phosphodiester at the terminus of a single strand, known (Fig. 2) to be a preferred substrate for the enzyme. But, because heating has no effect on duplex substrates made with a tyrosine-oligonucleotide, we prefer an alternate possibility: that heating serves to partially unfold the topoisomerase so as to enhance steric access to the scissile bond. Eukaryotic topoisomerase I is well known to be subject to a wide variety of covalent modifications (22). Perhaps one or more of these serves to loosen the enzyme structure when it is covalently trapped on DNA or even target it for proteolysis. A requirement for such modification would serve to insure that the phosphodiesterase does not act on covalent complexes that occur as transient intermediates during the normal operation of topoisomerase I.

The scheme of Fig. 6 also makes obvious the logic for future investigations of the biological role of the phosphodiesterase. For each of the proposed steps, one can use a combination of biochemical and genetic methods to ask how many enzymes of each type are in a cell, to establish their identity and to inquire about their relevance for cellular homeostasis. Of special interest for future work is the way the proposed pathway might influence the sensitivity of a cell to agents like camptothecin that increase the burden of covalently trapped topoisomerases (5, 6, 27). Cells with genetic alterations in their sensitivity to this type of drug are known (refs. 28–30 and references therein); these may be of use in sorting out the significance of the repair pathway that we propose. Moreover, camptothecin derivatives are promising anti-cancer agents (31); it is to be expected that better understanding of how cells deal with trapped topoisomerases can only improve the effectiveness with which they are used.

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